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Two Mechanisms of Allelic Complementation Among Tryptophan Synthetase Mutants of *Saccharomyces cerevisiae*

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Two different types of allelic complementation were observed in tryptophan synthetase mutants of the yeast *Saccharomyces cerevisiae*. Each type is associated with a different mechanism for the enzymatic conversion of indole-3-glycerol phosphate (InGP) to tryptophan. Mechanism I is utilized by a hybrid tryptophan synthetase that resembles, but is not identical with, the wild-type enzyme. Mechanism II is due to a sequential conversion of InGP to free indole, and indole to tryptophan. Two partially active mutant enzymes rather than a single hybrid enzyme catalyze the sequential reaction steps. This is an example of intracellular cross-feeding. The quantitative evaluation of mechanism II leads to the conclusion that tryptophan synthetase in yeast is most likely a dimer of two identical subunits.

Genetic complementation as a criterion for functional independence between recessive mutations remains a useful and indispensable tool for genetic analysis in spite of some conspicuous conceptual and practical shortcomings. The assumption that mutants affected in different genes will complement each other, whereas those affected in the same gene will not, is not always justified. Two familiar exceptions, for example, are (i) the noncomplementing behavior of some strong polar mutants with nonallelic mutants affected in the same operon, and (ii) complementation among allelic mutations by formation of hybrid enzymes.

These complications are not usually serious when complementation tests are used to classify mutants among clearly different loci. When used to study the structural organization of complex loci, however, complementation will yield useful information only when the mechanism of the observed complementation is understood. It is evident that within functionally complex gene-enzyme systems several mechanisms of complementation can occur (4).

Mutations affecting the enzyme tryptophan synthetase in several microorganisms have been extensively analyzed, genetically and biochemically (1, 5, 7, 9). These studies have revealed differences in the structural organization of the enzymes, and presumably, in the organization of the corresponding genes.

Tryptophan synthetase, in all microorganisms in which it has been studied, catalyzes the following three reactions: (A) indole-3-glycerol phosphate (InGP) $\rightleftarrows$ indole (In) + glyceraldehyde-3-phosphate (GAP); (B) In + L-serine $\rightarrow$ L-tryptophan (Trp); (C) InGP + L-serine $\rightarrow$ Trp + GAP. Pyridoxal phosphate (PALP) is required as a coenzyme in reactions B and C. In *Escherichia coli* and in *Neurospora crassa* it has been demonstrated that indole is not an obligatory intermediate in the formation of tryptophan from InGP (3, 9, 10). Reaction C, therefore, is considered to be the one of physiological importance. But in spite of the general similarities in their reaction mechanisms, the enzymes from these two organisms and their corresponding genes are organized quite differently. In *E. coli*, the enzyme is composed of two readily separable, nonidentical subunits, which correspond to two adjacent genes (A gene and B gene; 9). Mutation in the A gene results in loss of activity in reactions A and C, and mutation in the B gene results in loss of activity in reactions B and C (9). In *Neurospora*, however, the enzyme does not readily dissociate into subunits, and the mutations exhibit a more complex relationship to the enzymatic activities. Most mutants have lost all three reactions, and only a small minority retains activity for reactions A (indole accumu-

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lators) or B (indole utilizers; 9). Furthermore, the *Neurospora* mutants exhibit a complex pattern of allelic complementation; there is a large class of mutants that do not complement with any other tryptophan synthetase mutants.

In the yeast *Saccharomyces cerevisiae*, the tryptophan synthetase system is quite similar genetically to that in *Neurospora*. The same general types of mutants are found, and the same general pattern of complementation among them is observed. In the yeast system, however, it is possible to identify most of the totally noncomplementing mutants as nonsense mutants, by virtue of their suppressibility by supersuppressors (7). Failure of these nonsense mutants to complement is not necessarily evidence that they are in a single gene, owing to the possibility that they are in different genes in a single operon and are exhibiting strong polarity. On the other hand, the complementation between certain pairs of mutants does not prove that they are affected in different genes unless it can be established that the complementation results from formation of normal wild-type tryptophan synthetase.

For a preliminary investigation of the mechanism of complementation in the tryptophan synthetase locus (tr_5) in yeast, we selected two pairs of mutants that complement strongly. One pair consists of an indole-accumulating mutant and an indole-utilizing one. The other pair consists of two mutants that lack all three activities and are affected near opposite ends of the genetic map of the locus. Our experiments reveal the existence of two distinct mechanisms of complementation, neither of which involves normal wild-type enzyme. The two mechanisms of complementation involve different enzymatic mechanisms for catalyzing conversion of InGP to tryptophan.

MATERIALS AND METHODS

Yeast strains. The standard wild-type diploid strain (X2180) used in these studies arose as a sporulating diploid in a culture of the standard haploid strain S288C (R. K. Mortimer, *personal communication*).

The following mutants of the structural gene for tryptophan synthetase (tr_5locus) were studied: $tr_{5\text{-}7}$, $tr_{5\text{-}11}$, $tr_{5\text{-}18}$, $tr_{5\text{-}67}$, and $tr_{5\text{-}36}$. The isolation and some of the properties of these mutants have been described previously (7). The genotypes and phenotypes of the particular strains used are given in Table 1. To simplify the descriptions of the experiments we shall designate different tr_5 mutations by their allele numbers. IA or IU preceding a number will identify the mutant as an indole-accumulator or indole-utilizer. Figure 1 is a genetic map constructed by the X-ray method (8) showing the positions of these mutations within the tr_5 locus. Several other mutations are included for reference; $tr_{5\text{-}27}$ is the most proximal known mutant, and $tr_{5\text{-}29}$ is the most distal, relative to the centromere on chromosome VII; $tr_{5\text{-}6}$ and $tr_{5\text{-}26}$ are additional indole-accumulating mutants, and $tr_{5\text{-}66}$ is another indole-utilizer.

Hybrid diploid strains were obtained by mass-mating overnight haploid cultures on YEPD-agar medium (*see* below) and isolating individual zygotes by micromanipulation. Heteroallelic and heterozygous diploids are designated by the allele numbers of their parents, using "+" to designate the wild-type tr_5 allele derived from S288C.

Media. The culture media used for these strains have been described previously (7). The complex medium (YEPD), containing 1% Difco yeast extract, 2% Difco peptone, and 2% dextrose, was supplemented with 80 mg of adenine and 80 mg of L-tryptophan per liter. Synthetic medium lacking tryptophan is designated −TR.

Preparation of crude extracts. Cells were grown aerobically to the plateau of the growth curve in liquid YEPD. Crude extracts were prepared by disrupting the cells in a French pressure cell (Aminco Instrument Corp., New York, N.Y.) at 20,000 psi, as described previously (T. R. Manney, Genetics, *in press*). Usually, crude extracts diluted to a protein concentration of 20 mg/ml were used for determination of enzyme activities without further treatment. When indicated, the extracts were dialyzed against 1,000 volumes of buffer containing 0.1 M potassium phosphate buffer and 0.01 M potassium ethylenediaminetetraacetate (EDTA) at *p*H 7.2. Dialysis was carried out at 4 C for 2 hr, and the buffer was changed at the end of the first hour.

Growth rates. Complete growth curves were determined in liquid −TR synthetic medium with a Klett-Summerson colorimeter equipped with a green filter. Cultures (50-ml) in 250-ml Erlenmeyer flasks were inoculated at a density of approximately 5×10^6 cells/ml (14 to 18 Klett units) and incubated on a

FIG. 1. *Map of tr_5 mutants determined by X-ray method (8).*

TABLE 1. *Genotypes and phenotypes of tr_5 mutants*

Strain	tr_5 Allele	Genotype	Phenotype[a]
JC20	7	*a* $tr_{5\text{-}7}$ $ad_{2\text{-}1}$	A^- B^- C^-
MA14	11	*α* $tr_{5\text{-}11}$ $ad_{2\text{-}2}$ le_1	$A^\pm$ $B^\pm$ C^-
XD7-S19	IA18[b]	*a* $tr_{5\text{-}18}$ $ad_{2\text{-}1}$ me_1	A^+ B^- C^-
MG16	IU67[c]	*α* $tr_{5\text{-}67}$	A^- B^+ C^-
MD40	36[d]	*α* $tr_{5\text{-}36}$ $ad_{2\text{-}1}$ me_1	A^- B^- C^-

[a] A, B, and C refer to activities in tryptophan synthetase reactions.
[b] Indole-accumulating mutant.
[c] Indole-utilizing mutant.
[d] Nonsense mutant.

rotary shaker at 30 C. Growth rates were determined graphically from the exponential portion of the growth curves.

Enzyme assays. The procedures described previously (T. R. Manney, Genetics, *in press*) were used to determine the activity of tryptophan synthetase in the three reactions, with the following change: the assay mixtures for reactions A and C contained 0.4 μmole of InGP-*2*-^{14}C at a specific activity of 0.3 μc/μmole.

The formation of free indole as an intermediate in reaction C was measured by carrying out the conversion of ^{14}C-InGP to tryptophan in the presence of unlabeled indole. The reaction mixture contained 0.4 μmole of labeled InGP at a specific activity of 2.8 μc/μmole, 0.4 μmole of indole, 100 μmoles of potassium phosphate buffer, 10 μmoles of potassium-EDTA, 60 μmoles of DL-serine, 1.5 μmoles of PALP, and crude extract in a total volume of 1.0 ml at *p*H 7.2. The reaction tubes were incubated for 30 min at 37 C, transferred to boiling water for 2 min, and then cooled in ice. The amounts and radioactivity of indole and tryptophan were determined by the procedure described by DeMoss (3).

Protein determination. Protein concentrations were estimated by the method of Lowry et al. (6). Crystalline bovine plasma albumin (Armour and Co., Chicago, Ill.) was used as a standard.

RESULTS

Complementation and cross-feeding. The degree of complementation that can be observed among the mutants studied depends on the criterion used to measure it. Striking differences were observed when complementing diploids were compared with wild-type strains by growth rate in liquid medium, by time for colony formation, or by specific activity of cell-free extracts.

Table 2 summarizes the results of quantitative comparisons of complementing hybrid diploids with heterozygous and homozygous wild-type diploids. For both of the complementing diploids, the exponential growth rate was approximately 80% of that of the wild-type despite the fact that they contained less than 10% of the wild-type tryptophan synthetase activity (InGP → Trp). This is consistent with the heterozygous wild type (+/36) having a growth rate of 90% of that of the homozygous wild type, but only approximately one-half the specific activity of tryptophan synthetase. Similarly, when streaked or replica-plated onto −TR agar, all four diploid strains grew up overnight. However, when dilute suspensions (<1,000 cells/plate) were plated on the same medium, the behavior of the complementing diploids was strikingly different. The 7/11 diploid formed visible colonies within 2 days as did both wild-type strains. The IA18/IU67 diploid, however, did not form colonies for at least 4 or 5 days.

TABLE 2. *Relative growth rates and specific activities of wild-type and complementing diploids*

Strain	*trs* Genotype	Relative exponential growth rate[a] on liquid −TR	Relative specific activity[b] on reaction C (InGP → Trp)	Time for formation of visible colonies on −TR agar
				days
X2180	+/+	1.00	1.00	2
XD9	+/36	0.88	0.43	2
XD24	7/11	0.77	0.07	2
XD15	IA18/IU67	0.80	0.06	4–5

[a] Measured exponential growth rate divided by exponential growth rate of X2180 (0.51 generation/hr).

[b] Specific enzyme activity divided by specific activity in X2180 (1.4×10^{-3} units/mg of protein).

In addition to complementation, IA18 can promote some growth of IU67 merely by cross-feeding. Whenever both mutants were streaked near each other on −TR agar, growth of IU67 was pronounced near the streak of IA18 and decreased with increasing distance from the indole-accumulating cells, indicating that the amount of indole excreted by IA18 was sufficient to support growth of IU67. We observed such cross-feeding only in combinations of an indole-utilizing mutant with an indole-accumulating one.

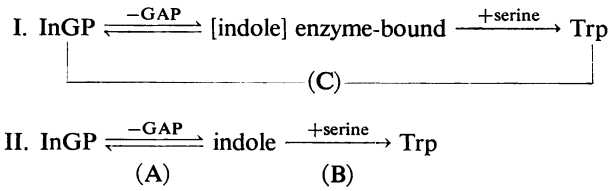
The striking difference in their growth properties suggests that these two complementing hybrids produce quite different forms of tryptophan synthetase. The existence of three distinct enzymatic activities for the enzyme provides a way to qualitatively characterize mutant enzymes, independently of the amount of enzyme formed, and thereby to compare their properties with wild-type and other mutant strains. Throughout the range of enzyme concentrations at which the assays are linear, the ratios among the activities for any form of the enzyme are independent of the specific activity. Accordingly, we used the ratios of the activity in reaction A to that in reaction B for qualitative characterization of the tryptophan synthetases formed by different strains.

Specific activities of wild type. The specific activities of the haploid and diploid wild-type strain in the three tryptophan synthetase reactions are given in Table 3. Under the conditions used, the conversion of indole to tryptophan was the most active reaction. The specific activities were equal in both the haploid and diploid strains. This result is in agreement with the findings of O. Ciferri, S. Sora, and O. Tiboni (*personal communication*) that the specific activity of tryp-

tophan synthetase is the same for haploid, diploid, and tetraploid homozygous wild-type strains, and is proportional to the gene dosage in heterozygotes. They also demonstrated that the diploids and tetraploids contain, respectively, two times and four times the amount of enzyme per cell as haploids.

Relative specific activities of mutants and heterozygous diploids. Table 4 gives the relative specific activities of extracts from mutants and from complementing diploids and of mixtures of mutant extracts. Each value is expressed as the fraction of the wild-type diploid specific activity for the same reaction. In addition, the ratios of the absolute values of the specific activities for reactions A and B are shown. None of the mutants showed measurable activity in reaction C (InGP → Trp), except 11, which also had approximately 6% of wild-type activity in reaction A (InGP → In) and 0.4% in reaction B (In → Trp). However, IU67 was as active as wild type in reaction B, and IA18 had approximately 40% of wild-type activity in reaction A. In extracts of the complementing diploids 7/11 and IA18/IU67, the activities in reaction C were slightly less than 10% of the wild type. However, in spite of having equal reaction C activities, their activities in reactions A and B differed widely, as illustrated by the A/B ratio. This difference was primarily the consequence of the abnormally high ratio in extracts of 7/11, and it further demonstrated the difference between the enzymes made by these two strains and their difference from the wild-type enzyme.

Mixtures of extracts from mutants 7 and 11 revealed no complementation in vitro, whereas under the same conditions a mixture of IA18 and IU67 extracts had considerable activity in reaction C. Since the specific activities (Table 4) are based on the total amount of protein in the mixtures, the values for reactions A and B are approximately 50% of those of the mutant extracts themselves. In contrast, the specific activity for reaction C in this mixture is nearly two times that found in the corresponding IA18/IU67 diploid. The absence of activity in the mixture of 7 and 11 suggests that hybrid tryptophan synthetase, which presumably is formed in vivo, is not formed in vitro under the conditions used in this investigation. If it is, therefore, assumed that no hybrid is formed in the mixture of IA18 and IU67, the formation of tryptophan can only be explained as the sum of reactions A and B. This suggests the occurrence of two reaction mechanisms.



In mechanism I, which is utilized in reaction C of the wild-type tryptophan synthetase, indole remains bound to the enzyme. Mechanism II involves indole as a free intermediate and would be anticipated if the two half reactions were catalyzed by different enzyme molecules. Mechanism II, therefore, could account for the complementation observed in IA18/IU67.

Analysis of reaction C mechanism. The important difference between these two mechanisms lies in the formation of free indole in mechanism II, but not in mechanism I. To determine the fate of the indole moiety during the conversion of InGP to tryptophan, we incubated ^{14}C-InGP, labeled in the 2 position of the indole ring, in a complete mixture for reaction C, with added unlabeled indole. The specific radioactivities of indole and tryptophan after incubation provide a quantitative measure of the amount of free indole formed during the reaction. These experiments are similar to those reported by DeMoss (3) confirming that free indole is not an obligatory intermediate in reaction C with the *Neurospora* enzyme. Our experiments differed, however, in that reaction C was only slightly inhibited by indole in the *Saccharomyces* enzyme, but was totally inhibited in *Neurospora* tryptophan synthetase (3).

TABLE 3. *Specific activities[a] of wild-type tryptophan synthetase*

Strain	Reaction A (InGP → In)	Reaction B (In → Trp)	Reaction C (InGP → Trp)	A/B
X2180 (diploid)	0.135 × 10^{-3}	3.32 × 10^{-3}	1.37 × 10^{-3}	0.04
S288C (haploid)	0.128 × 10^{-3}	3.23 × 10^{-3}	1.41 × 10^{-3}	0.04

[a] The specific activities are expressed as units of enzyme/mg of protein. Enzyme activity is defined in international units, i.e., 1 unit catalyzes the conversion of 1 μmole of substrate per min under the conditions used.

TABLE 4. *Relative specific activities[a] of mutants, complementing diploids, and in vitro combinations of mutant extracts*

Strain	*trs* Genotype	Reaction A[b] (InGP → In)	Reaction B (In → Trp)	Reaction C (InGP → Trp)	A/B (absolute values)
X2180	+/+	1.00	1.00	1.00	0.04
S288C	+	0.94	1.08	1.05	0.04
JC20	7	0.01	<0.001	<0.001	
MA14	11	0.06	0.004	0.005	
XD7-S19	IA18	0.39	<0.001	<0.001	
MG16	IU67	<0.001	1.02	<0.001	
XD24	7/11	0.20	0.04	0.07	0.20
XD15	IA18/IU67	0.20	0.38	0.06	0.02
JC20 + MA14	7 + 11	0.03	0.001	0.002	
XD7-S19 + MG16	IA18 + IU67	0.23	0.41	0.11	0.02

[a] The activities are expressed as fractions of wild-type diploid specific activities (reaction A: 0.135×10^{-3} units/mg of protein; reaction B: 3.32×10^{-3} units/mg of protein; reaction C: 1.37×10^{-3} units/mg of protein).

[b] Dialyzed extracts.

The following three possible situations could be anticipated: (i) tryptophan formed by mechanism I only, (ii) tryptophan formed by mechanism II only, or (iii) tryptophan formed by both mechanisms simultaneously. In the first case, the addition of cold indole should have no effect on the reaction kinetics. The formation of radioactive tryptophan should be proportional to enzyme concentration and to time of incubation, and there should be no radioactive indole formed. In the second case, the addition of cold indole should have a profound effect on the reaction kinetics. The formation of radioactive tryptophan should be proportional to the square of the enzyme concentration and should also be a nonlinear function of the time of incubation. Radioactive indole would be formed during the reaction at a rate depending on the relative rates of reactions A and B. In the third case, in which both mechanisms operate, the reaction kinetics should be of a mixed type. The formation of radioactive tryptophan should follow the sum of a linear and a second-order relationship with enzyme concentration and with time of incubation. Radioactive indole would be formed.

The kinetics of formation of radioactive tryptophan and indole from ^{14}C-InGP for the wild-type enzyme, for the two complementing diploids, IA18/IU67 and 7/11, and for a mixture of the haploid extracts IA18 and IU67 are illustrated in Fig. 2 and 3.

Figure 2A shows the amount of ^{14}C-tryptophan formed from ^{14}C-InGP in the presence of ^{12}C-indole during a 30-min incubation with different amounts of crude extracts. Clearly, two different types of curves are found. In wild-type and in 7/11, the amount of ^{14}C-tryptophan formed is proportional to the amount of protein,

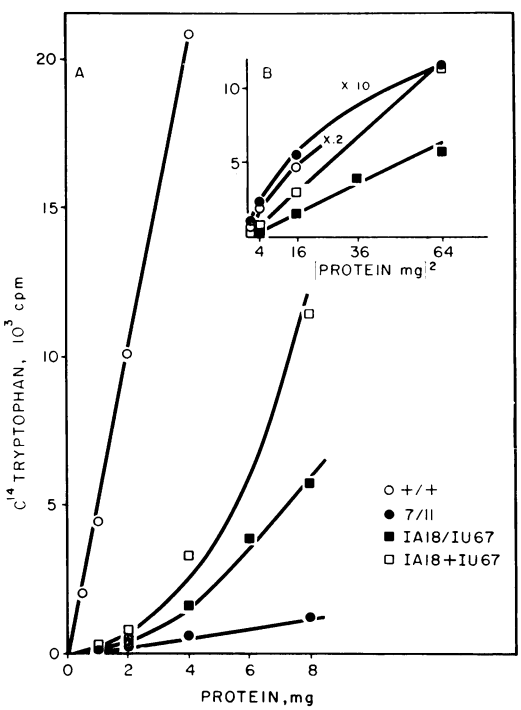


FIG. 2. *(A) Formation of ^{14}C-tryptophan from ^{14}C-InGP in the presence of unlabeled indole by crude extracts from wild-type (+/+) and complementing diploids (7/11 and IA18/IU67) and by a mixture of IA18 and IU67 extracts containing equal amounts of protein from each haploid extract (IA18 + IU67). Reaction mixtures containing 0.4 μmole of ^{14}C-InGP (4.5×10^5 counts per min per μmole) and 0.4 μmole of ^{12}C-indole were incubated for 30 min at 37 C. (B) Values from (A) plotted against the square of the amount of protein. Values for wild type are multiplied by the factor 0.2 and values for 7/11 by the factor 10.*

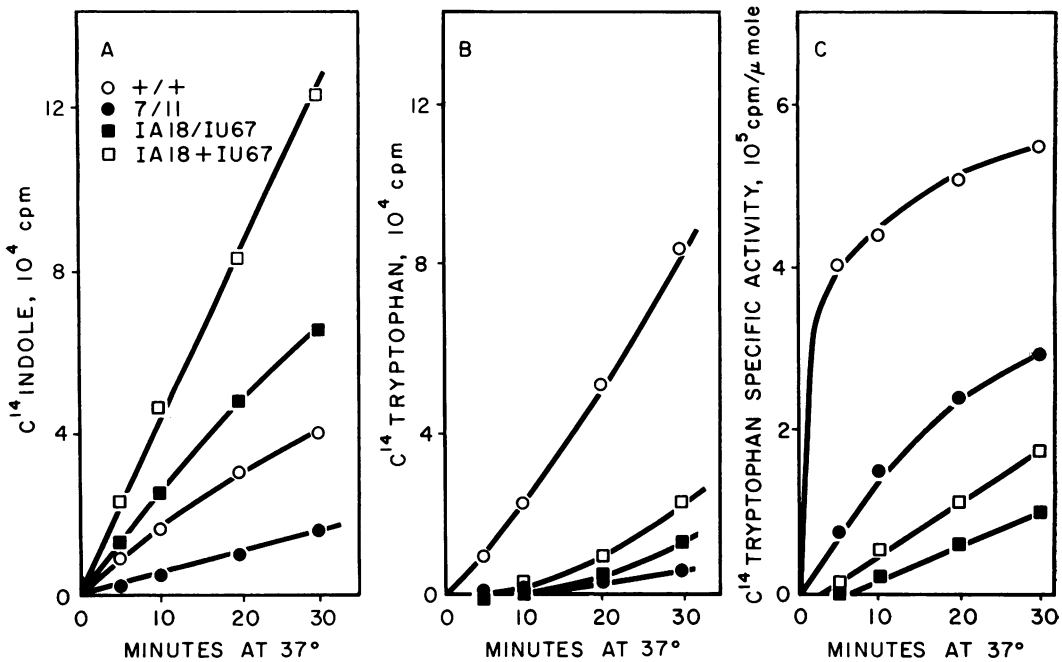


FIG. 3. *Kinetics of ^{14}C-tryptophan formation from ^{14}C-InGP in the presence of ^{12}C-indole with crude extracts from wild-type (+/+) and complementing diploids (7/11 and IA18/IU67) and with a mixture of IA18 and IU67 extracts (IA18 + IU67). Reaction mixtures contained 4 mg of protein, 0.4 μmole of ^{14}C-InGP (4.3 × 10^6 counts per min per μmole) and 0.4 μmole of ^{12}C-indole as substrates. (A) Formation of ^{14}C-indole; (B) formation of ^{14}C-tryptophan; (C) specific activity of tryptophan.*

consistent with the predictions of mechanism I. In a mixture of IA18 and IU67 extracts and in the extract of the corresponding diploid IA18/IU67, the amount of labeled tryptophan is a second-order function of the amount of protein (Fig. 2B), suggesting that two independent, sequential reaction steps leading to the formation of tryptophan from InGP are involved (mechanism II).

Figures 3A and 3B show the time course of indole and tryptophan formation from labeled InGP. All of the enzyme preparations tested formed significant amounts of ^{14}C-indole (Fig. 3A), and in all cases except the wild type the amount of labeled indole exceeded the amount of labeled tryptophan formed. That this free indole is an intermediate in tryptophan formation is illustrated by the nonlinearity of the ^{14}C-tryptophan-formation curves (Fig. 3B). Even the wild-type enzyme exhibited significant nonlinearity in this test, although the curve for wild-type enzyme in Fig. 2 appears to be linear. These results strongly suggest that mechanism II is involved to some extent in all four cases, although it appears as only a minor component of the reaction for the wild-type enzyme. Apparently mechanism I, in which free indole is not an intermediate, is the predominant reaction mechanism in *Saccharomyces* tryptophan synthetase, as it is in *Neurospora* and *E. coli*.

The formation of labeled indole and the nonlinear appearance of labeled tryptophan provide sensitive criteria for the operation of mechanism II. The relative contributions of the two mechanisms, however, are more readily appreciated from a plot of the specific radioactivity of the tryptophan formed as a function of time. This type of plot (Fig. 3C) reveals that with the 7/11 enzyme, as with the wild type, label appears in tryptophan without any apparent lag, whereas with the IA18 and IU67 combinations there is a lag of 3 to 5 min before radioactivity appears in tryptophan. Furthermore, with the IA18 and IU67 combinations the specific radioactivity of tryptophan increases linearly with time, after the first few minutes, indicating that tryptophan is formed from a single precursor, which is becoming increasingly labeled. In contrast, the rate of increase of the specific activity of the tryptophan formed by the wild-type and 7/11 extracts decreased with time. This can be explained by the simultaneous formation of tryptophan from two precursors, the labeled InGP (reaction C) and the initially unlabeled indole

(reaction B). Since a significant amount of unlabeled tryptophan is formed by reaction B, the specific radioactivity of the product does not reach that of the substrate. However, during the course of the reaction the indole pool becomes increasingly labeled by reaction A so that the specific radioactivity of tryptophan continues to increase.

These results clearly demonstrate that the enzyme formed by complementation in 7/11 is different from wild-type tryptophan synthetase, but nonetheless it is able to convert InGP directly to tryptophan. They further demonstrate that complementation, in vivo and in vitro, between IA18 and IU67 does not result in any activity capable of direct conversion. Both the diploid extract and the mixture appear to employ only mechanism II.

Analysis of reaction mechanism II. Complementation by reaction mechanism II would not require the formation of a hybrid enzyme at all, as indicated by the fact that the mutant strain IA18 accumulates enough indole in the growth medium to support some growth of the mutant strain IU67. This fact by itself, however, does not rule out the possible existence of a hybrid enzyme utilizing mechanism II.

To determine the type of interaction between these two mutant enzymes we examined the effect of varying proportions of the two mutant extracts on tryptophan formation under normal conditions (no added indole). In each case, the amount of one extract was held constant in the reaction mixture (at 0.5 mg of protein) and the amount of the other was varied. The rate of tryptophan formation in these reaction mixtures is shown in Fig. 4, curves A and B. In both cases, the curves initially rise linearly with increasing extract and finally plateau. When the mixture is saturated with the extract from the indole-accumulating mutant (curve B), the tryptophan forming activity is nearly twice as great as when it is saturated with the extract from the indole-utilizing one. In both cases, there is substantial increase above the activity obtained with equal amounts of the two extracts.

If the observed activity were the result of a hybrid formed by an equal number of subunits, the two curves should plateau at the same activity. Although the levels observed could conceivably reflect the formation of a hybrid containing twice as many subunits from the indole-utilizing mutant, it seems more likely that the indole-accumulating mutant enzyme is producing indole that the indole-utilizing one is converting to tryptophan. Different saturation levels would be expected as a consequence of

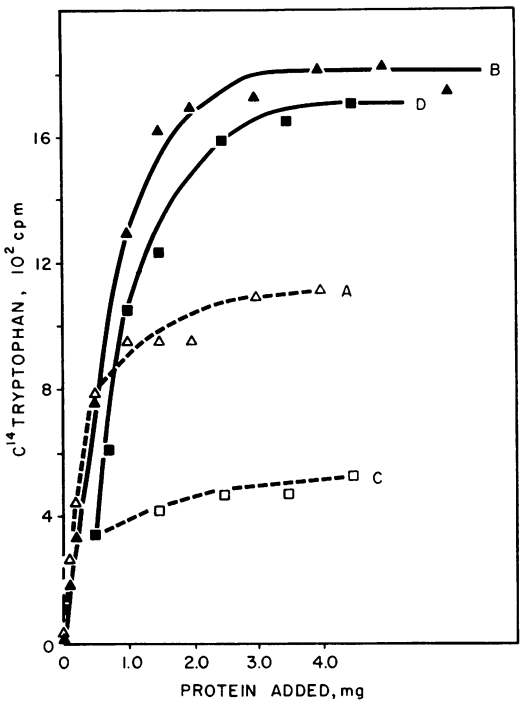


FIG. 4. *In vitro complementation in mutant extracts IA18 and IU67 and diploid extract IA18/IU67. Reaction mixtures containing a fixed amount of one extract and different amounts of another were incubated for 30 min at 37 C. The formation of ^{14}C-tryptophan from ^{14}C-InGP (0.4 μmole, 4.5 × 10^5 counts/min per μmole, per reaction mixture) was measured; curve A, 0.5 mg of IA18 protein, different amounts of IU67 protein; curve B, 0.5 mg of IU67 protein, different amounts of IA18 protein; curves C and D, 1.0 mg of IA18/IU67 protein, different amounts of IU67 and IA18 protein, respectively. The values for the diploid extract were plotted as if it contained 0.5 mg of each parent extract.*

the different properties of the two enzymes, under the conditions of the reaction. In curve A, the increased activity in the presence of excess indole-utilizing extract most likely reflects the reversibility of reaction A (InGP $\rightleftarrows$ In). The excess indole-utilizing activity would pull the reaction toward indole by removing the indole faster, and thus result in an increase in the overall rate. The much higher saturation level in curve B, in which the indole-utilizing extract is limiting, could result from the substrate concentration dependence of reaction B (In $\rightarrow$ Trp). In the mixtures containing lower amounts of indole-accumulating extract, the indole concentration is probably below the level necessary to allow reaction B to proceed at its maximal rate. Increasing the amount of indole-accumulating extract increases the indole concentration.

Consequently, the reaction rate increases until a saturating concentration of indole is reached.

The characteristics of these saturation curves provided a way to examine the composition of the extract formed by the IA18/IU67 hybrid diploid. If this extract contained equal amounts of the same mutant enzyme forms found in extracts of the parent haploids, it should produce the same saturation curves when assayed in the presence of an excess of either of the two haploid extracts (Fig. 4, curves C and D). The abscissa points are plotted on the assumption that extract from the diploid containing a total of 1.0 mg of protein is equivalent to a mixture of mutant extracts containing 0.5 mg of protein from each. The results reveal that this assumption is not at all justified. First, the activity of the extract from the diploid is only about 50% of that found in the equivalent mixture of the two haploid parent extracts (Table 4). Second, the discrepancy is much greater for the indole-accumulating activity than for the utilizing activity. In fact, curves C and D correspond more closely to the curves that would be predicted if the diploid extract is equivalent to a 2:1 mixture of indole-utilizing and indole-accumulating extracts.

These results can be explained by a simple model. We assume that these mutant enzymes are dimers of identical subunits, and that the number of subunits formed is proportional to gene dosage. A_2 and B_2 represent the enzymes made by the indole-accumulating and indole-utilizing mutants, respectively, and AB represents a hybrid dimer. If dimers are formed at random in the hybrid, one would expect a distribution of $1A_2$: $2AB$:$1B_2$. Then, if it is further assumed that the hybrid dimer, AB, is only active in reaction B (In $\rightarrow$ Trp), with half as much activity per molecule as the B_2 dimers, the hybrid extract would indeed contain a 2:1 mixture of the two activities, as well as the correspondingly reduced overall activity.

DISCUSSION

These studies illustrate that the qualitative phenotype of tryptophan independence can reflect a variety of forms of the enzyme tryptophan synthetase. Detailed analysis of the enzyme mechanisms involved in the catalytic activities of two complementing diploids reveals that apparently allelic mutants may interact at quite different levels of molecular organization. The level of interaction obviously depends on the nature of the altered gene products, but it also appears to depend on the general organization of the enzyme.

The two mutants 7 and 11 have lost all but traces of the three important tryptophan synthetase activities. Complementation in a hybrid of these two mutants apparently results from formation of a hybrid enzyme, which is similar to wild-type tryptophan synthetase, but clearly distinguishable. The two partially active mutants IA18 and IU67, on the other hand, retain nearly wild-type activity for reactions A and B, respectively. Complementation in a hybrid of these two, however, can be entirely explained by intracellular cross-feeding between the two half-reactions. It is not necessary to assume the existence of a hybrid enzyme to account for the enzyme activities, but quantitative considerations strongly suggest that a hybrid may nonetheless be formed.

The results of these experiments lead us to the following conclusions about the organization and activity of *Saccharomyces* tryptophan synthetase. These conclusions are provisional by virtue of the indirect nature of the evidence.

Subunit structure. The simplest interpretation of these results follows from the assumption that tryptophan synthetase in *S. cerevisiae* is composed of two identical subunits. The most likely alternative hypothesis would be that it consists of two nonidentical subunits, one possessing the active site for reaction A and the other having the active site for reaction B. This would be analogous to the organization of *E. coli* tryptophan synthetase (2). Although no critical test of either of these models was performed, the results are not consistent with the hypothesis of nonidentical subunits unless additional, more complicated assumptions are made.

The results clearly demonstrated that the tryptophan synthetase activity in the IA18/IU67 hybrid is drastically different from the wild-type enzyme, and the specific activity rules out the possibility that any significant amount of wild-type enzyme is made by this strain. To reconcile this with two active sites residing on different polypeptide chains would require the additional assumption that the pairs of subunits corresponding to each homologous chromosome always occur together in the same enzyme multimer. Thus, if the tr_5 locus consists of a two-gene operon, then it must be assumed that the polypeptide chains translated from each two-gene messenger ribonucleic acid molecule become associated before they mix with the molecules translated from other messengers. Formally, this is equivalent to having a single gene, making a single kind of polypeptide chain, and the two models are not distinguishable by complementation experiments. The one-gene model is simpler, and, in absence of any experimental precedence

for the other type of interaction, we prefer the simpler model for now.

Mechanisms of complementation. The results with these two complementing diploids demonstrate two mechanisms of allelic complementation. One probably involves the formation of a hybrid enzyme, but the other is not dependent on formation of an active hybrid. The second mechanism, in effect, is a simple case of intracellular cross-feeding of an indole-utilizing capability by an indole-accumulating activity. In this relatively well-defined enzymatic system, the two mechanisms are easy to distinguish. In many systems, however, in which little if anything is known of the enzymology, and especially if an intermediate is not excreted by the accumulating mutant, these mechanisms would be indistinguishable. Since the cross-feeding mechanism does not depend on the properties of a hybrid enzyme, it cannot provide any information about the requirements for activity in such hybrids. We believe that the existence of this mechanism poses a serious obstacle to the detailed interpretation of complementation maps in terms of active hybrid formation, if the biochemical mechanisms are not understood.

Tryptophan synthetase mechanism. Whereas the results for the wild-type enzyme demonstrate that the predominant mechanism for tryptophan formation from InGP does not require formation of free indole, the data demonstrate that a significant amount of free indole is formed in the reaction. The contrast between this finding and the results cited for other organisms (3, 9, 10) indicates that, although the general mechanisms of tryptophan synthetase are quite similar in different organisms, there are at least quantitative differences in the enzymatic properties. In this connection, it should be observed that the differences in the growth properties of the two complementing diploids, in spite of their very similar reaction C specific activities, demonstrate a distinct biological advantage of mechanism I compared with mechanism II. Although the two hybrids employing different mechanisms have indistinguishable exponential growth rates, the one that uses mechanism II has an abnormally long lag period when growing from a single cell.

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